

A METHOD FOR REMOVING TRANSMISSIBLE LYSINS FROM SECONDARY CULTURES OF BACTERIA¹

R. C. THOMAS,

The Ohio Agricultural Experiment Station,
Wooster, Ohio

INTRODUCTION

Precursors of transmissible lysins or bacterial viruses may exist for a given bacterium wherever organic matter is found. The writer has been particularly interested in such sources as seed and grain extracts, crop residues, and the sap of living plants (Thomas 5, 6, 7). Since the addition of grain or plant extracts to primary cultures of bacteria, and also in some instances to secondary cultures, starts the transmissible lysis phenomenon, it seems reasonable to assume that the lytic factor might be removed and the original culture reclaimed if suitable methods could be devised for that purpose.

In a number of instances, bacteria have been rendered apparently free of the lysins associated with them. The method used is outlined in this paper.

It was thought advisable in this preliminary investigation to work only with known cultures. This means that the primary culture and source of the lysin in each instance, were known. The species of bacteria used in this investigation were assumed to be primary species. A comparison of the types of growth of a culture of bacteria, with and without a lysin present, in the same kind of liquid medium furnishes valuable information and enables one to judge from the character of growth of an unknown isolate, whether or not a lytic factor is present. So long as a transmissible lytic factor is in intimate contact with a culture, the secondary growth which develops is no longer susceptible to the same lysin, because usually in a secondary growth, the lytic factor continues to develop with the organism indefinitely. The presence of a lysin can be demonstrated by testing the bacteria-free filtrate of a secondary culture against the primary or original organism. These secondary cultures are usually considered to be resistant to lytic action. Such cultures are so saturated with the lysin that the addition of more has no effect. The lysin-organism relationship of secondary cultures has never been satisfactorily explained.

The possibility of removing a transmissible lysin from a bacterium would depend upon the kind of affinity or relationship which exists between the two—whether the combination is fixed or reversible. A host-parasite relationship might be expected to be irreversible and there could not be much hope of bringing about a separation. On the other hand, if the lytic condition is due to an acquired enzyme or nucleoprotein, a separation may be feasible. In any case, it would be necessary to find an adsorbent which would have at least as great, if not greater, affinity for the lytic substance than its homologous bacterium.

Early investigators of nucleic acids discovered that these acids have a remarkable property of removing proteins from solution. The combination of albumin and syntonin with various nucleic acids was studied by Milroy (4). Evidence seems to indicate that the viruses are at least in part protein. Tobacco mosaic virus is thought to be a nucleoprotein. It was therefore decided to determine what effect nucleic acids might have upon the lytic factor bacterium combination of different species of bacteria and to find out if an organism could be rendered again susceptible to lysis by removal of the virus by adsorption on nucleic acid.

¹Published with the approval of the Director of the Ohio Agricultural Experiment Station.

METHODS OF INVESTIGATION

Nucleic acids are available commercially from only a few different sources. Preparations from yeast, wheat germs and thymus were obtained. Since these acids are insoluble in water it was necessary to add an alkali to effect solution. For this purpose either sodium hydroxide or sodium bicarbonate solution was used. The preparation from animal tissue was furnished as a sodium salt and was adjusted by the addition of dilute acetic acid pH 6.50 to 7.00.

Other extracts containing nucleic acid from alfalfa, timothy, oats and potato were prepared by digesting 50 grams of the finely ground material with 5 per cent ammonium hydroxide. Extraction was continued for about three hours, with frequent stirring, on a water-bath heated to 70°C. The liquid was then removed by filtering, the ammonia neutralized with hydrochloric acid and the precipitate which formed was collected by centrifuging. The crude extract was redissolved with ammonium hydroxide and reprecipitated twice, then washed to remove ammonium chloride, and finally suspended in 50 milliliters of distilled water. A few drops of sodium hydroxide were added to bring the suspension into solution. These crude extracts were used without further attempts at purification.

A 2 per cent solution of each commercial nucleic acid was prepared. Whatever micro-organisms were present were removed by filtering through a sterile Selas candle. One milliliter of the filtrate was added to 8 milliliters of sterile nutrient broth or distilled water. Then, each tube containing sodium nucleate was inoculated with one drop, approximately $\frac{1}{2}$ of a milliliter, of a secondary culture containing a transmissible lysin. After thoro mixing the preparation was allowed to stand at room temperature for periods varying from 1 to 12 hours. The period of contact of the lytic factors with the different cultures varied from 2 weeks to 3 months. This did not seem to be of much importance. If it was at all possible to free some of the organisms of a culture from the lysin the time of previous contact or association was of little significance.

At varying intervals, after a culture had been in contact with a nucleate, dilution plates were poured, using nutrient agar. These plates were incubated for 3 or 4 days at 28°C. Then single colonies were selected and transfers made to tubes of broth. Dilution plates were made also of the original culture which did not contain a lytic substance. Using the type of colony growth found in these plates as a guide, similar colonies were picked from the plates prepared from the secondary culture treated with sodium nucleate. Usually only surface colonies, with regular or entire margins showing a smooth type of growth, were selected. If the primary culture developed a pigment, then only colonies showing the same shade of color were chosen for transfer.

After incubation for 24 hours a comparison of the growth of the new isolates with that of the parent culture furnished a good indication if any had been selected which were lysin-free. Colonies still containing a lysin either failed to make any growth or developed only a slight clouding of the broth. The isolates which grew normally were next tested against the original virus filtrate. If they showed the same susceptibility to lysis as the primary culture, they were assumed to be virus-free. Cultures of isolates which still retained the virus were not susceptible to lysis by the original lytic factor. The presence of a lytic factor in an isolate which shows only limited growth can be demonstrated readily by testing the filtrate of such a culture against the parent culture or against those isolates from which the factor has been removed. This procedure can be used for analyzing a species of bacteria which may be suspected of harboring a virus, thus rendering it virus-free and of learning the nature of the original form from which the secondary strain was derived.

RESULTS

In table I is shown a number of species of bacteria for which transmissible lysins were developed from known sources and from which they have been removed. The criterion of lysin removal is based on the fact that the new isolates, following treatment with a sodium nucleate, regained susceptibility to the original lysin. So long as a lytic factor was in association with a secondary culture it did not show any evidence of lysis.

In nearly all cases, secondary cultures developed from the bacterial species recorded in table I retained their lysins in symbiotic relationship regardless of how many transfers were made in such media as nutrient broth or agar. One exception was *Xanthomonas pruni* which could be rendered lysin-free by merely plating in nutrient agar. This could not be done with any of the other secondary cultures without previous contact with some kind of nucleic acid or plant extract. All of

TABLE I
NUCLEIC ACIDS AND PLANT EXTRACTS ARE USEFUL AS ADSORBENTS OF TRANSMISSIBLE LYSINS

Organism	Source of lysin	Source of adsorbent	Period of contact with adsorbent	Number of isolates	Number reacting with original lysin
<i>Xanthomonas juglandis</i>	Oat extract.....	Thymus.....	9 hrs.	6	2
<i>Xanthomonas stewartii</i>	Oat extract.....	Wheat germ....	12 hrs.	12	10
<i>Erwinia carotovora</i>	Oat extract.....	Wheat germ....	3 hrs.	6	1
<i>Corynebacterium sepeidonicum</i>	Potato vine.....	Potato extract	2 hrs.	18	10
<i>Corynebacterium michiganense</i>	Oat extract.....	Wheat germ....	4 hrs.	6	4
<i>Xanthomonas pruni</i>	Oat extract.....	Wheat germ....	3 hrs.	6	6
<i>Escherichia coli</i>	Animal.....	Wheat germ....	1 hr.	6	5
<i>Escherichia coli</i>	Animal.....	Wheat germ....	2 hrs.	6	5
<i>Escherichia coli</i>	Animal.....	Wheat germ....	7 hrs.	6	0
<i>Staphylococcus aureus</i>	Human.....	Wheat germ....	2 hrs.	4	2
Unidentified species AC.....	Oat extract.....	Wheat germ....	1½ hrs.	8	7
Unidentified species 1A.....	Alfalfa extract...	Wheat germ....	3 hrs.	5	5
Unidentified species 1A.....	Clover extract...	Wheat germ....	3 hrs.	5	5
Unidentified species 1A.....	Timothy extract...	Wheat germ....	3 hrs.	5	0
<i>Agrobacterium tumefaciens</i> ...	Oat extract.....	Thymus.....	4 hrs.	12	5
<i>Agrobacterium tumefaciens</i> ...	Oat extract.....	Timothy.....	4 hrs.	6	6
<i>Agrobacterium tumefaciens</i> ...	Oat extract.....	Alfalfa.....	4 hrs.	6	6
<i>Agrobacterium tumefaciens</i> ...	Oat extract.....	Oat grain.....	4 hrs.	6	4

the nucleic acid salts and plant extracts used as lysin adsorbants give Feulgen's reaction. Agar-agar also gives this reaction. Probably there is sufficient adsorbing material in agar-agar to remove the lytic factor from *Xanthomonas pruni*.

There does not seem to be much correlation between the source of lytic factors and the kind of nucleic acid used to remove them. Thymus nucleic acid was effective for the lysins of *Xanthomonas juglandis* and *Agrobacterium tumefaciens*, both of which came from a plant source. Nucleic acid from wheat germs removed lytic factors from *Escherichia coli* and *Staphylococcus aureus*, the lysins being of animal origin in both cases.

The only instance of strict specificity is found with *Corynebacterium sepeidonicum*. The lysin for the organism had come from an extract of a dead potato vine which had succumbed to the ring-rot disease. An extract of potato tuber only was found to be of value for adsorbing the lysin of that organism and thus restoring it to its primary form and rendering it again susceptible to lysis by the original lytic factor.

The necessary period of contact of a secondary culture with the nucleic acid varied with the organism, and the optimum time could be determined only by trial platings at stated intervals. Those which yielded best results are recorded for each culture in table I. For *Escherichia coli* a contact period of one or two hours before a sample was removed for plating gave virus-free colonies, but 7 hours were too long. Nucleic acids and plant extracts prevent the growth of some species of bacteria indefinitely while other species start to grow sooner or later. Growth of *Escherichia coli*, before the 7-hour period of contact would account for the multiplication of the virus and also for the fact that no colonies could be obtained susceptible to lysis at that time.

Nucleic acids from four different sources were effective for adsorbing lysin from a secondary culture of *Agrobacterium tumefaciens*. When a secondary culture of this organism, without previous contact with a nucleic acid, was plated on nutrient agar, no colonies were ever found free of the lytic factor.

Cultures of virus-free bacteria killed by heating at 60°C. for 30 minutes were used instead of nucleic acid in order to determine if the lytic particles would be fixed on the dead bacteria. No organisms from which the lytic factor had been separated were ever obtained by platings from such preparations.

The removal of a lytic factor from a culture, thus restoring it to susceptibility to lysis and to normal growth in ordinary media, does not furnish assurance that the reclaimed culture is identical with the original in all respects. In order to gain further information in this connection, plant inoculation was tried. Tomato plants were inoculated from cultures of *Agrobacterium tumefaciens* with and without a lysin present and also with isolates from which the lytic factor had been removed and which had again become susceptible to lysis.

The presence of a lytic factor developed from oat grain extract in a culture of *Agr. tumefaciens* prevented that organism from developing galls when inoculated into tomato plants. After the lysin had been removed by treatment with timothy extract, the culture was again rendered susceptible to lysis and also regained capacity to produce galls.

DISCUSSION

Many species of bacteria, particularly primary forms, acquire lytic factors readily when they come into contact with the juices of higher plants, seed extracts and plant residues. The presence of a lytic factor in a culture somehow changes the metabolic activity of the organism. Evidence of this is indicated by failure of the secondary culture to grow in the same medium as the primary, by the need for vitamin or amino acid amendments and by change in color.

With a more definite understanding of the sources of lytic factors it is proper to raise the question regarding any recognized species of bacteria, whether it is a primary or secondary form. This consideration may have a definite bearing upon our viewpoint of classification and nomenclature in the future. If we can take a primary organism and develop a number of different variants or strains with the aid of plant or seed extracts our conception of what constitutes a species or a strain will be brought more definitely into focus.

Secondary forms of bacteria may be anticipated in fermentation processes where the substrate is seeds, grains or plant material which have not been thoroly sterilized by heat.

The fact that nucleic acids are effective in removing transmissible lytic factors from bacteria, thus permitting the organisms to develop as they did before a lysin was added to a culture, suggests that a class of enzymes known as nucleases may enter into lysin formation. A nucleic acid might be expected to have greater affinity for a nuclease than for other enzymes or proteins.

Very little is known regarding the nucleases, particularly their action on living substrates. Ribonuclease was prepared from commercial pancreatin by Dubos and Thompson (1). Kunitz (3), using different methods, isolated the same enzyme in crystalline form from the same source. This particular nuclease readily attacks the sodium salt of yeast nucleic acid. Greenstein (2) reports that thymonucleodepolymerase occurs not only in animal but also in plant tissues. Extracts of the embryos of corn, wheat, pumpkin, sunflower and lima bean exert a strong depolymerizing effect upon sodium thymonucleate. Apparently the distinction between plant and animal nucleic acids is rapidly disappearing. This fact is also evident in the general lack of specificity among nucleic acids in separating lytic factors from bacteria.

SUMMARY

Transmissible lytic factors can be removed from bacteria and the organisms restored to their original form.

Organisms which have been rendered lysin-free again become susceptible to lysis by the original lytic factor. In the case of *Agrobacterium tumefaciens*, the recovery of the capacity to induce gall formation in tomato was demonstrated following treatment with timothy extract.

The method of procedure outlined in this study can be applied to a culture which may be suspected of having a transmissible lysin associated with it. The parent primary culture frequently can be recovered.

LITERATURE CITED

1. Dubos, Rene J. and R. H. S. Thompson. The decomposition of yeast nucleic acid by a heat-resistant enzyme. Jour. Biol. Chem. 124: 501-510. 1938.
2. Greenstein, Jesse P. Ribonuclease and thymonucleodepolymerase. National Cancer Inst. Proc. Part II, Vol. 1: 113. 1942.
3. Kunitz, M. Crystalline ribonuclease. Jour. Gen. Physiol. 24: 15-32. 1940.
4. Milroy, T. H. Ueber die Eiwiss-Verbindungen der Nucleinsäure und Thyminsäure und ihre Beziehung zu den Nuclein und Paranuclein. Zeitschrift für Physiol. Chemie (Hoppe-Seyler's) 22: 307-326. 1896.
5. Thomas, R. C. Transmissible lysins in water extracts of seeds. Science 88: 56-57. 1938.
6. ——— Additional facts regarding bacteriophage lytic to *Aplanobacter stewartii*. Phytopath. 30: 602-611. 1940.
7. ——— The bacteriophage reaction for the identification of bacteria. Ohio Agr. Exp. Sta. Tech. Bull. 11, pp. 1-12. 1947.